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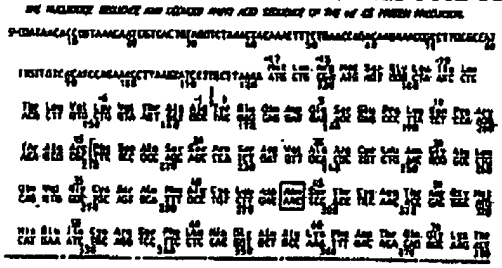

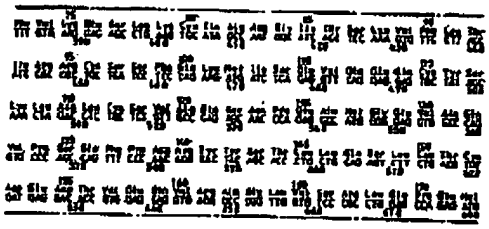

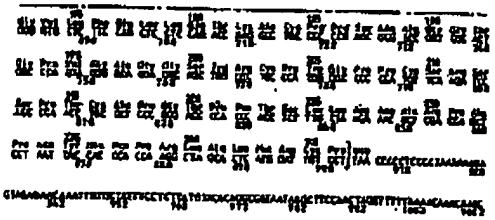

(57) Abstract

Essentially pure CS protein, a protein isolated from the Corpuscles of Stannius having ion-transfer properties, precursors thereof and sub-units or fragments thereof. A gene encoding the CS protein and precursors thereof.

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THE CS PROTEIN OF THE CORPUSCLES OF STANNIUS

This invention relates to the isolation and characterisation of the CS protein from the Corpuscles of Stannius. The invention is also concerned with the molecular cloning and characterisation of the gene sequence encoding the CS protein.

Note: References referred to hereafter are collected at the end of the description.

In 1839 Stannius described yellow gland-like structures scattered through the kidney of the sturgeon and noted that these structures also occurred in Teleost fishes as two distinct small bodies and suggested that these organs might represent the adrenal glands of fishes. This homology with the adrenal gland of mammals was accepted until more extensive embryological studies were undertaken by Huot (1898) who described the differing origins of the Corpuscles of Stannius and the adrenal cortex. However, confusion still persisted. Giacomini in 1908 (a and b) described a mass of tissue in the anterior region of the kidney which on the basis of morphological characteristics resembled the interrenal system which he designated as the anterior interrenal as distinct from the

Stannius corpuscles which he designated as the posterior interrenal. Giacomini also made the observation that the corpuscles were present only in those animals where the parathyroids were absent. Vincent (1898) at about the same time had shown that the removal of the Corpuscles of Stannius was not fatal and therefore assumed they were not essential for life. However Petit (1896) had demonstrated that the removal of one corpuscle resulted in compensatory hypertrophy of the remaining one, thus suggesting that the organ might be an endocrine gland. Following these early observations, the function of the Corpuscles of Stannius has fascinated biologists continuously to 1986 and numerous studies have broached this issue.

The corpuscles from various fishes have been extensively examined morphologically both at the light and electron microscopy level (Vincent, 1898; Oliveseau and Fontaine, 1965; Krishnamurthy and Bern, 1969; Kistow and Piepho, 1963; Wendelaar Bonga and Greven, 1975; Wendelaar Bonga et al. 1980 and Bhattacharyya et al. 1982). There is a concensus view that they contain at least one type of secretory granule. Variations with life cycle, with level of maturation, with age and sex as well as species differences have created a confused picture.

Overall, ablation experiments have led to the view that the Corpuscles of Stannius may have an osmoregulatory role, however their close association with the kidney leave open the possibility of surgical damage to the delicate renal area, which may have compounded the issue of function. There is some evidence that they may have a parathyroid hormone like role in calcium metabolism (Millet et al. 1980 and 1981; Lopez et al. 1984), a prolactin-like action in the gill (Ogasaware and Hirano, 1984) and possibly be the source of a renin-like enzyme capable of angiotensin II production (Chester Jones 1966;

Ogawa and Sokabe, 1982).

Infusion of extracts of Corpuscles of Stannius have been reported variously as having actions on water, sodium, calcium, phosphate and magnesium metabolism.

The secretory product of the corpuscles of Stannius has until now remained unresolved.

SUMMARY OF THE INVENTION

The CS protein of the Corpuscles of Stannius has been purified to homogeneity. The partial protein sequence of the purified CS protein was obtained and used to construct synthetic oligonucleotides which were in turn used to isolate DNA encoding the CS protein of the Corpuscles of Stannius. Characterization of this DNA shows that it encodes the CS protein and a precursor thereof comprising an N-terminal prohormone segment and a signal peptide sequence which are attached to the CS protein sequence.

The precursor of the CS protein having an N-terminal prohormone segment and signal peptide is hereinafter referred to as the prepro CS protein. The precursor of the CS protein having an N-terminal prohormone segment is hereinafter referred to as the pro CS protein.

Isolation of the CS protein from the Corpuscles of Stannius will enable further investigations to be carried out on the biological activity of this protein.

According to one aspect of the present invention, there is provided essentially pure CS protein.

According to a further aspect of the present invention, there is provided the prepro CS protein, the pro CS protein or the signal or prohormone peptides of the prepro CS protein. The invention is also directed to sub-units or fragments of the prepro CS protein.

According to a still further aspect of the present invention there is provided a gene encoding the prepro CS protein or sub-units or fragments thereof. Particularly,

there is provided DNA sequences which encode the pro CS protein, the CS protein or the signal or prohormone peptides of the prepro CS protein.

The gene encoding the prepro CS protein or sub-units or fragments thereof may be inserted into appropriate transfer vectors. Suitable vectors include bacterial plasmids, yeast plasmids, phage and other viral vectors.

The gene encoding the prepro CS protein or sub-units or fragments thereof may be inserted into appropriate expression vectors for subsequent expression of the prepro CS protein or sub-units or fragments thereof in eurkaryotic or prokaryotic host cells.

Suitable prokaryotic host cells include various E. coli species, Pseudomonas and Serratia marcenes.

Suitable eurkaryotic host cells include filamentous fungi, various strains of yeast and mammalian cells (both primary and cell lines).

The isolation of DNA encoding the prepro CS protein and sub-units or fragments thereof will enable large amounts of the corresponding protein products to be produced by recombinant DNA methodology.

According to a yet further aspect of the present invention, there is provided a method for the production of the CS protein, said method comprising the steps of:

(a) inserting the gene encoding CS protein into an expression vector;

(b) transfecting a cell with the expression vector of step (a); and

(c) culturing the cell containing the expression vector to produce the CS protein and subsequently recovering said protein.

As the protein sequence of the prepro CS protein has been elucidated by recombinant DNA techniques, the prepro CS protein and fragments or sub-units thereof may be

produced using conventional protein synthesis techniques (Barany and Merrifield, 1980).

We have synthesized a peptide fragment of the CS protein precursor corresponding to amino acids 16 to 35 of Figure 3, to investigate the physiological role of this protein. Studies utilising this peptide indicate that the CS protein affects ionic activity in vivo, particularly, in increasing sodium excretion and decreasing plasma potassium concentration. This activity is characteristic of the CS protein, and has been exemplified by, but is in no way restricted to a sheep model. Studies we have carried out in fish indicate this protein is also active in decreasing calcium uptake.

The CS protein and peptide fragments thereof have potential as therapeutic agents in the treatment of cardio-vascular disease, renal disease and electrolyte disorders, particularly, oedema, heart failure and high blood pressure. In addition to the above, the N-terminal peptide fragment of the CS protein precursor corresponding to amino acids 16 to 35 of Figure 3, and other fragments of the CS protein, may be useful tools in determining physiological control mechanisms in the kidney, and the control of blood pressure.

According to another aspect of the present invention, there is provided a therapeutic composition comprising the CS protein or peptide fragments thereof in association with a pharmaceutically acceptable carrier or excipient. Such compositions may be useful in the treatment of cardio-vascular disease, renal disease and electrolyte disorders.

According to a further aspect of the present invention, there is provided a method for the treatment of cardio-vascular disease, renal disease and electrolyte disorders, comprising the administration of a

therapeutically effective amount of the CS protein or peptide fragments thereof, either alone or in association with a pharmaceutically acceptable carrier or excipient.

The isolation and characterization of DNA encoding the prepro CS protein enables labelled DNA or RNA probes to be prepared. These probes, which may contain all or part of the DNA encoding the prepro CS protein, can be used to identify homologous or similar sequences in higher organisms, including man.

DNA probes may be labelled isotopically using for example ^{32}P or ^{125}I. DNA probes may also be labelled with biotin or avidin, fluorescent or chemiluminescent reagents, or other appropriate labelling molecules.

RNA probes may be produced from the DNA encoding the CS protein using, for example, the SP6 vector system (Pharmacia Corporation, Piscataway, N.J.).

It is well established that the genetic code contains redundancies, that is, certain amino acids are coded for by more than one codon. Accordingly, the invention includes DNA encoding the prepro CS protein or sub-units or fragments thereof where the natural codons are replaced by other codons which code for the same amino acid.

According to a further aspect of the present invention, there is provided a method for the isolation of the CS protein comprising:

(a) detergent extraction of the Corpuscles of Stannius;
(b) electrophoretic separation of the detergent extract in a gel matrix; and
(c) electroelution of the separated CS protein from step (b) and recovery of the purified CS protein.

According to a still further aspect of the present invention, there is provided a method for the isolation of a gene encoding the prepro CS protein comprising the steps of:

(i) preparing a hybridization probe based on the protein sequence of the prepro CS protein;

(ii) screening a cDNA library prepared from mRNA isolated from the Corpuscles of Stannius with the probe; and

(iii) identifying and isolating those DNA sequences from the cDNA library of step (ii) which hybridize to the probe.

DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a polyacrylamide gel electrophoresis profile of the isoelectric focusing gel section (pI 5.7 - 6.5) of the CS protein after staining with Coomassie Blue;

FIGURE 2 is the sequencing strategy for the CS protein mRNA:

A. Schematic diagram of the CS protein mRNA. The 5' and 3' untranslated regions are indicated by the solid line. The hatched box represents the signal peptide sequence, the solid box represents the prosegment, and the open box represents the mature protein.

B. The arrangement of the four positive CS protein cDNA clones. λCS.1 was isolated from the RNAse H cDNA Library and λCS.2, λCS.4 were isolated from the S1-nuclease library.

FIGURE 3 shows the nucleotide sequence and deduced amino acid sequence of the eel CS protein precursor;

FIGURE 4 shows a hybridization histochemical analysis of the eel Corpus Stannius-kidney region. 6μm sections of eel CS-kidney region after probing with ^{32}P labelled 75 mer oligonucleotide. The CS region is shown in the lower left region of the autoradiograph.

FIGURE 5 shows the effect of renal arterial infusion into 7 conscious sheep of the N-terminal 25 amino acid fragment of the CS proteins (peptide 'U'). Peptide 'U' was infused at a rate of 50 μg/h. Graph (A) is a plot

of urinary sodium excretion (μmol/min) against time (hours). Graph (B) is a plot of plasma potassium against time (hours).

Definitions:

The term "CS protein" used herein refers to a polypeptide isolated from the Corpuscles of Stannius, having a molecular weight approximately between 28,000 and 42,000 Daltons as determined by SDS-PAGE and an amino acid sequence substantially corresponding to amino acids 16-246 of Figure 3.

The amino acid sequence of the CS protein depicted in Figure 3 may be varied by the substitution, addition or deletion of one or more amino acids. Variants which possess ion-transfer activity, characteristic of the CS protein, are included within the term "CS protein". Such variants may be produced by solid phase peptide synthesis techniques (Barany and Merrifield, 1980). Alternatively, genetic engineering techniques such as site directed mutogenesis (Botstein and Shortle, 1985), restriction endonuclease digestion, and the ligation of DNA fragments, (Maniatis et al., 1982) may be employed to construct DNA expression vectors which express variants of the CS protein.

We have isolated the CS protein from the eel; Anguilla australis. It is to be understood that the CS protein may be readily isolated from other animal species following the teaching of the present application. Any such protein having a molecular weight approximately between 28,000 and 42,00 Daltons and an amino acid sequence exhibiting substantial homology with amino acids 16-246 of Figure 3 is to be included within the definition "CS protein".

Substantial homology when used in the above context refers to at least 75% homology with amino acids 16-246 of

Figure 3.

The term "pro CS protein" used herein, refers to a protein having a sequence corresponding to amino acids 1-246 of Figure 3.

The term "prepro CS protein" used herein, refers to a protein having a sequence corresponding to amino acids -17-246 of Figure 3.

"Essentially pure" when used to define the CS protein produced by the present invention refers to the CS protein substantially free of protein or other materials ordinarily associated with the Corpuscles of Stannius, ordinarily greater than or equal to 95% of the total protein being CS protein by weight.

The term "sub-unit" or "fragment" when used in relation to the CS protein refers to a peptide having an amino acid sequence which is included within the amino acid sequence of the CS protein. Peptides having more than six amino acids are likely to be unique to the CS protein. In order to test whether a peptide is unique to the CS protein, its amino acid sequence may be compared with amino acid sequences on record in amino acid sequence data banks such as the EMBL Data Base (compiled by the European Molecular Biology Laboratory), the Dayhoff Data Base or the Gene Bank Data Base (compiled by the National Institutes of Health, U.S.A.). Sub-units or fragments of the CS protein may or may not possess biological activity.

The term "natural codons" refers to those codons which naturally encode amino acids of the prepro CS protein.

The term "sub-unit" or "fragment", when used in relation to genes encoding the prepro CS protein, refers to a DNA or RNA sequence included in the gene encoding the prepro CS protein. A fragment may comprise single or double stranded DNA or RNA, and is generally in excess of 10 nucleotides. The sequence of any such fragment may be

compared with sequences recorded in any of the data banks mentioned above to establish whether it is unique to the CS protein.

The following terms have the meanings set forth below:

DNA	-	deoxyribonucleic acid
RNA	-	ribonucleic acid
cDNA	-	complementary DNA (enzymically synthesized from a mRNA sequence
mRNA	-	messenger RNA
A	-	Adenine
T	-	Thymine
G	-	Guanine
C	-	Cytosine
U	-	Uracil
SDS-PAGE	-	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis

EXAMPLES:

EXAMPLE A - Tissue Extraction and Isolation of the CS Protein

(i) Tissue Extracts

Corpuscles of Stannius and samples of several other tissues (kidney, atria, ventricle, muscle and liver) were obtained from decapitated eels. These tissues were placed immediately on dry ice and stored under liquid nitrogen until used. A modified Baier technique (1984) was used for homogenation of the tissues. The zwiterionic detergent CHAPS was substituted for Nonidet P40. The tissue homogenates were centrifuged in an Eppendorf (Trade Mark) centrifuge for 3 minutes and the supernatant used immediately for electrophoresis analysis.

(ii) Two dimensional gel electrophoresis techniques

This was performed essentially as originally described by O'Farrell (1975) except that CHAPS was substituted for

Nonidet P40 as above, and for the electrofocussing gels, the urea solution was deionized just prior to use. After electrofocussing, the gels were equilibrated in O'Farrell's buffer "O" for 10 minutes and then run in the second dimension. These procedures revealed major proteins of the corpuscles of Stannius which are not present in other eel tissues. The portions of the CS electrofocussing gels (pI 5.7 - 6.5) were cut out, equilibrated and run in the second dimension as before. The line of spots containing the CS protein was visualized with 4 M sodium acetate (Higgins and Dehmus, 1979) or Coomassie Blue (CBB) stained (Figure 1).

Once identified the spots were punched out, the gel disc was washed in water and stored at -20°C until electroelution. Electroelution was performed at 5°C according to the technique of Hunkapillar et al (1983) in an electroelution dialysis cell (Caltech).

(iv) Characteristics of the CS Protein

The eluted protein was harvested and precipitated overnight with 9 volumes of cold methanol. Following centrifugation at 5°C, the pellet was washed with a small amount of cold methanol, air dried, dissolved in 0.05% SDS and reprecipitated as above. The air dried pellet was then stored in a dessicator at 5°C until analysed. Electrophoretic analysis has established that the CS protein has a native molecular weight of 42,000, and under reducing conditions a major component of 32,000.

The following results were obtained on analysis of the isolated CS protein:

1. Treatment with N-Glycanase demonstrated that the 32,000 monomer is N-linked glycosylated either at a -Asn-X-Thr or -Asn-X-Ser (where X is any amino acid except Pro) position and that the molecular weight was reduced to 28,000 following removal of the

carbohydrate.

2. Amino acid analysis was performed according to standard procedures on a Beckman System 6300 Amino Acid Analyser.

An approximation of the ratio of the amino acid residues in the isolated CS protein (based on an assumed total number of amino acids of 210) gave the following results:

Asx (18), Thr (10), Ser (17), Glx (25), Pro (10), Gly (21), Ala (17), Cys (present), Val (15), Met (5), Ile (14), Leu (21), Tyr (10), Phe (13), His (3), Lys (8), Arg (8), indicating a predominance of Glx (Glu or Gln), Gly, Leu, Asx (Asp or Asn), Ser, Ala and the presence of cysteine residues.

3. Amino acid sequencing

The following partial N-terminal sequence was obtained using an Applied Biosystems gas phase amino acid sequencer:

N-Phe-Ser-Ala-Ser-Ser-Pro-Ser-Asp-Val-Ala-Arg-x-Leu-Asn-Gly-Ala-Leu-Gln-Val-Gly-x-Ser-Ala-Phe-Ala-Leu--, where -x- respresents cysteine, threonine or possibly tryptophan.

EXAMPLE B - Isolation and Characterisation of a DNA Encoding the prepro CS Protein

(i) Synthetic oligonucleotide probes

An oligodeoxyribonucleotide probe was synthesized (using an Applied Biosystems DNA Synthesizer) corresponding to the predicted cDNA sequence of the amino terminal 25 amino acid sequence with the assumption that residues 12 and 21 were Thr and Trp respectively and using preferred codon choices for fish.

The 75-mer probe sequence was as follows:

5'-GGCGAAGGCGGACCATCCCACCTGCAGGGCTCCGTTCAGTGTTCTGGC
CACGTCGGATGGACAGGAGGCGGAGAA-3'

The 75-mer probe was 5' end labelled with ^{32}P and used to screen the cDNA clone bank constructed from mRNA of the Corpuscles of Stannius. This probe was also used for hybridization histochemistry studies.

(ii) Cloning and Sequence Analysis of cDNA for the Isolated CS Protein

Two different cDNA Libraries were constructed from eel CS poly(A)$^+$ RNA. The first library was derived from cDNA which was synthesized using standard procedures; oligo(dT)-primed AMV reverse transcriptase reaction followed by DNA polymerase and S_1-nuclease reactions (Maniatis et al, 1982). The second library was derived from cDNA synthesized using the RNase H procedure of Gubler and Hoffman (1983), which is known to produce full-length cDNA transcripts. Both cDNA libraries were cloned using the bacteriphage vector λgt 10 (Huynh et al, 1985).

Approximately 5,000 clones from each cDNA library were screened with the synthetic 75-mer oligonucleotide which was 5'-end labelled with [γ-^{32}P]ATP. Hybridization was carried out in 20% formamide, 50 mM $NaPO_4$ pH 6.8, 1mM Na pyrophosphate, 5 x SSC, 5 x Denhardts, 50 µg/ml salmon sperm. DNA at 42°C. Filters were washed (4 times) in 0.2 x SSC at 40°C. Three hybridization-positive clones were isolated from the S_1-nuclease cDNA library and one hybridization-positive clone was isolated from the RNase H library. Analysis of these clones by restriction endonuclease digests and Southern blotting revealed cDNA insert lengths of 0.32 (λCS.2), 0.8 (λCS.4) and 1.1 kb (λCS.3) for the

three S_1-nuclease clones and 2.2 kb for the one RNase H clone (λCS.1), as shown in Figure 2. Part A of Figure 2 represents a schematic diagram of the CS protein mRNA. The 5' and 3' untranslated regions are indicated by the solid line. The hatched box represents the signal peptide sequence, the solid box represents the prosegment, and the open box represents the mature protein. The nucleotide sequencing strategy and the linear arrangement of these clones is shown in Figure 2.

DNA fragments suitable for dideoxy chain termination sequencing (Sanger et al., 1977) were generated by sonication of the appropriate cDNA inserts (Deininger, 1983). These randomly generated fragments were subcloned into M13mp18. The nucleotide sequence of the CS protein cDNA is shown in Figure 3. The sequence of nucleotide residues 269-343 corresponds to the amino-terminal amino acid sequence region determined for the CS protein. It is interesting to note that the original 75-mer probe, designed using preferred codon choices, has 75% identity with the authentic sequence.

On the basis of methods for predicting secretory signal sequences (von Heijne, 1986), it is assumed that the site of cleavage between the signal peptide and the excreted protein is between the alanine and tyrosine residues, indicated by an arrow in Figure 3. This leaves a 15 amino acid pro-sequence extension at the amino terminus of the isolated CS Protein. The pro-sequence precursor is presumably processed by specific cleavage between the arginine and phenylalanine residues (at positions 15 and 16) to give the mature 231 amino acid CS protein shown in square brackets in Figure 3.

The deduced amino acid sequence of the eel CS protein has an Asn-Ser-Thr sequence at amino acids 44-46. The asparagine at position 44 is presumably the

carbohydrate attachment site. It is interesting to note that there are a total of 15 cysteine residues in the deduced sequence. If all the Cys residues are involved in cysteine bonds, this leaves an extra cysteine which could be free or linked to another peptide chain through a disulfide bond.

EXAMPLE C - Histochemical Localisation of mRNA Encoding the CS Protein

(i) Hybridization Histochemistry

This was performed on the Corpuscles of Stannius and other eel tissue, using the labelled 75-mer oligonucleotide probe as described by Coghlan et al. 1985. These studies revealed that the mRNA coding for this protein was located specifically in the Corpuscles of Stannius and not in any other eel tissues examined. Figure 4 shows specific labelling of the CS and not the kidney with the 75 mer probe.

EXAMPLE D - Synthesis and Biological Activity of a Fragment of the Pro CS Protein

(i) Synthetic Peptide Synthesis

The following peptides, corresponding to amino acids shown in Figure 3, were synthesized by solid-phase synthesis procedures (Barany and Merrifield, 1980) on an Applied Biosystems Model 430A Automated Peptide Synthesizer:

A	-1-15
B	1-15
C	16-35
D	96-109
E	118-151
F	217-246

Crude peptides were purified by gel filtration and preparative high performance liquid chromatography (HPLC)

under reducing conditions to homogeneity, as assessed by analytical HPLC and amino acid analysis. The cysteines were left unblocked and were tested for biological activity in the reduced monomeric form.

(ii) Biological Activity

Sheep were prepared surgically by removing the ovaries and the right kidney, the carotid artery was exteriorized and cannulas placed into the left renal artery and vein. Synthetic peptide was infused directly into the renal arterial cannula at 50 µg/hr (12 ml/hr) and urine was collected via a Foley bladder catheter. Blood samples were taken from the carotid artery. Urinary sodium and plasma potassium levels were determined by standard procedures. Only peptide C exhibited biological activity in this bioassay, and the results for this peptide are presented in Figure 5. All statistical analyses were by two-way analyses of variance. Each line in the Figure represents the data for an individual sheep (seven different sheep), and the shaded area shows the grouped data of one standard error of the mean on either side of the group mean. The results demonstrate that there is a small, but significant, increase in sodium excretion, and also a decrease in plasma potassium concentration in the sheep following infusion of a synthetic peptide fragment of the pro CS protein.

The above results indicate that the CS protein and fragments thereof, particularly corresponding to peptide C and peptides synthesized from around this region, have potential in the treatment of cardio-vascular disease, renal disease and electrolyte disorders; particularly oedema, high blood pressure and heart failure. Although these experiments have been carried out in sheep, it is to be understood that the therapeutic affect of these agents is not restricted to sheep.

(iii) An Alternative Assay for Biological Activity

The effect of peptide C, corresponding to amino acids 16-35 of Figure 3, on calcium uptake was tested in the assay described by Wagner et al., 1986. This assay measures inhibitory effects upon branchial calcium uptake and involves monitoring the rate of ^{45}Ca uptake by juvenile rainbow trout.

Results of this assay are shown in Table 1.

TABLE 1

Dose	(n)	Uptake	P	% Inhibition
Control (Saline)	7	2.62±0.44		--
10µg peptide C (4.37nM)	7	0.67±0.14		0.005 (-74%)
1µg peptide C (0.44nM)	8	1.25±0.19	P	0.01 (-53%)

These results indicate that the CS protein and a selected peptide fragment thereof have an inhibitory effect on calcium uptake.

(iv) Expression of the CS protein in Escherichia Coli

The CS protein cDNA has been cloned into an E. Coli secretion vector, in order to produce large quantities of the CS protein for structural and biological characterization. The CS protein cDNA clone λCS3 was inserted into the EcoR1 site of the vector ompA-2, a system developed by Ghrayeb et al. (1984). This secretion vector contains the coding sequence for the ompA signal peptide. This signal peptide guides the cloned gene product across the cytoplasmic membrane into the periplasmic space of the E.Coli cell. The signal peptide is then cleaved from the cloned gene

product by a specific signal peptidase. Thus, the gene product produced has the authentic amino terminus of the original protein.

The coding sequence for the ompA signal peptide was joined onto the coding sequence for the amino terminus of the mature CS protein, i.e. residue 16 in Figure 3, using the site-specific mutagenesis method described by Morinaga et al. (1984). This secretion vector containing the coding sequence for the ompA signal peptice/CS protein was designated pCS-2. Gel electrophoresis of proteins from E. Coli cells containing pCS-2, and Western blot aalysis using a CS protein antisera, indicate that the cells are producing a gene product which binds the CS protein antibody.

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SUBSTITUTE SHEET

CLAIMS:

1. Essentially pure CS protein.

2. Essentially pure pro CS protein.

3. Essentially pure prepro CS protein.

4. The signal or prohormone peptide of the prepro CS protein.

5. A peptide fragment of the pre pro CS protein.

6. A fragment as claimed in claim 5, which possess ion transfer activity characteristic of the CS protein.

7. A fragment as claimed in claim 6 having a sequence corresponding to amino acids 16 to 35 of Figure 3.

8. A therapeutic composition comprising the CS protein or peptide fragments thereof in association with a pharmaceutically acceptable carrier or excipient.

9. A method for the treatment of cardio-vascular disease, renal disease or electrolyte disorders, comprising the administration of a therapeutically effective amount of the CS protein or peptide fragments thereof either alone or in association with a pharmaceutically acceptable carrier or excipient.

10. A method for the isolation of the CS protein comprising:

(a) detergent extraction of the Corpuscles of Stannius;

(b) electrophoretic separation of the detergent extract; and

(c) electroelution and recovery of the separated CS protein from step (b).

11. A gene encoding the prepro CS protein.

12. A sub-unit of the gene claimed in claim 11, which encodes the pro CS protein, the CS Protein or the signal or prohormone peptides of the prepro CS protein.

13. A fragment of the gene claimed in claim 11, excluding those sub-units claimed in claim 12, which is unique to the gene encoding the prepro CS protein.

14. A transfer vector containing a gene, sub-unit or fragment as claimed in any one of claims 11 to 13.

15. A method for the isolation of a gene encoding the prepro CS protein comprising the steps of:

(i) preparing a hybridization probe based on the protein sequence of the prepro CS protein;

(ii) screening a cDNA Library prepared from mRNA extracted from the Corpuscles of Stannius with the probe; and

(iii) identifying and isolating those DNA sequences from the cDNA Library of step (ii) which hybridize to the probe.

16. A method for the production of the CS protein, said method comprising the steps of:

(a) inserting the gene encoding CS protein into an expression vector;

(b) transfecting a cell with the expression vector of

step (a); and

(c) culturing the cell containing the expression vector to produce the CS protein and subsequently recovering said protein.

17. A method as claimed in claim 16, wherein the expression vector is a viral vector.

18. A method as claimed in claim 16, wherein the vector is ompA-2 and the transfected cell is E. Coli.

19. The CS protein when produced by the methods of any one of claims 16 to 18.

20. The CS protein, methods for its preparation and pharmaceutical compositions containing it substantially as hereinbefore described.

21. A gene encoding the CS protein and methods for its production substantially as hereinbefore described.

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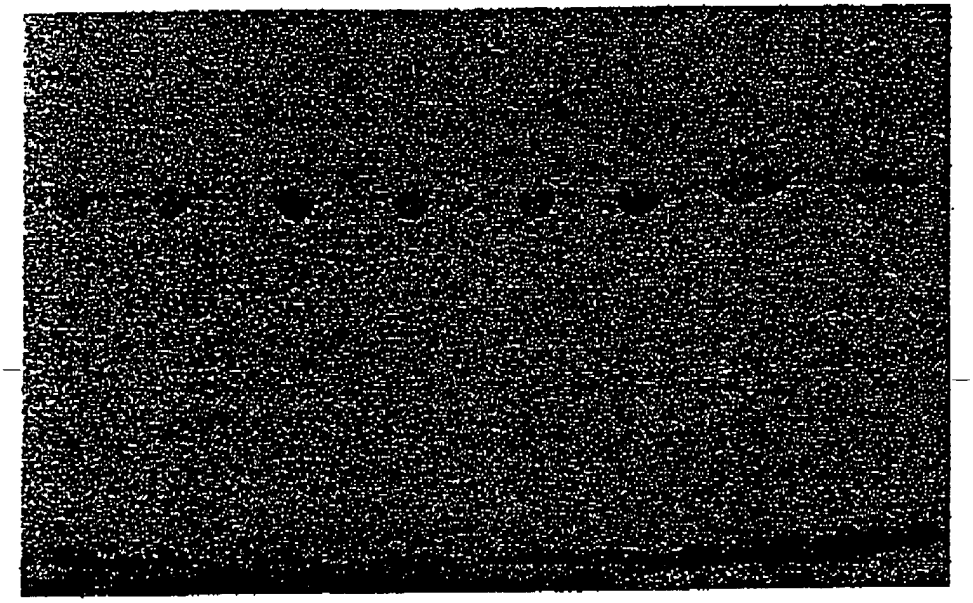

FIG. 1.

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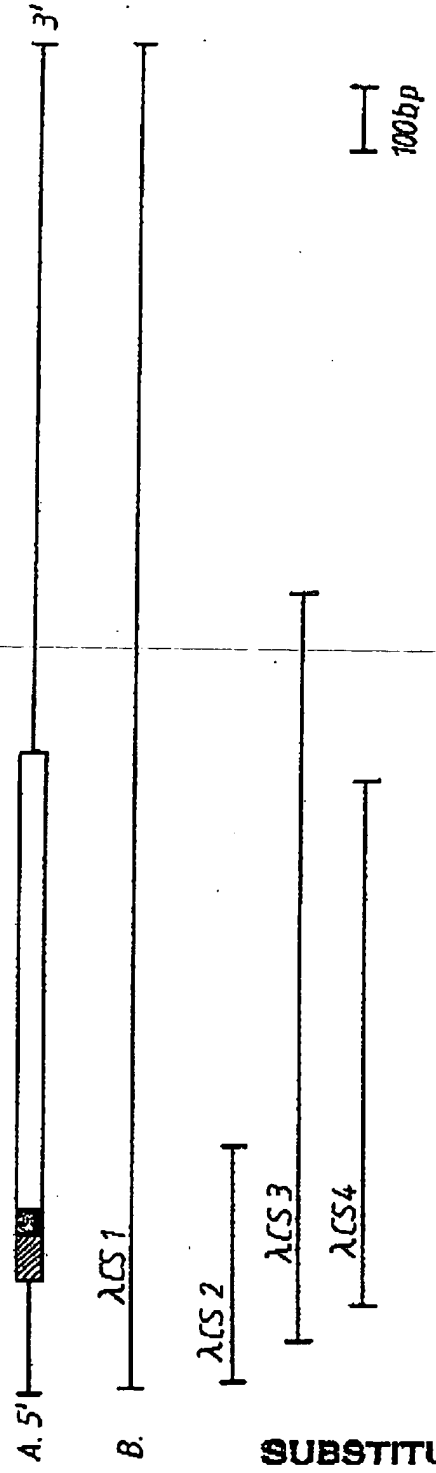

FIG. 2

THE NUCLEOTIDE SEQUENCE AND DEDUCED AMINO ACID SEQUENCE OF THE eel CS PROTEIN PRECURSOR.

```
5'-CGATAACACCGGTAAACAATCGTCACTGTAGGTCTAAACTACAAACTTTCTGAACCAGACAAGAAACCGCCTTGCGCCAT
          10        20        30        40        50        60        70        80

                                         -17      -15                 -10
                                         Met Leu Arg Met Ser Gly Leu Ile Leu
TTGTTGTCACATCCAGAAACCTTAAGTATCCTTGCTTAAAA ATG CTG CGA ATG AGT GGG CTA ATC CTC
        90        100       110       120         130           140

            -5                  -1  1               5                   10
Thr Leu Val Leu Val Thr Ala Ala Tyr Glu Gln Asp Glu Ser Glu Pro Leu Ser Pro Ary
ACG CTT GTG CTG GTA ACT GCT GCC TAC GAG CAG GAT GAG AGC GAG CCC TTA TCT CCA AGG
          158         168         178         188         198         208

        15              20                  25                  30
Thr Ala Arg [Phe Ser Ala Ser Ser Pro Ser Asp Val Ala Arg Cys Leu Asn Gly Ala Leu
ACA GCG CGC [TTC TCC GCC AGC AGC CCA TCT GAT GTT GCA CGC TGT CTG AAC GGG GCC CTG
          218         228         238         248         258         268

        35              40                  45                  50
Gln Val Gly Cys Ser Ala Phe Ala Cys Leu Asp [Asn] Ser Thr Cys Asn Thr Asp Gly Met
CAG GTG GGC TGC AGT GCA TTT GCC TGT CTT GAC [AAC] TCC ACC TGC AAC ACC GAC GGC ATG
          278         288         298         308         318         328

        55              60                  65                  70
His Glu Ile Cys Arg Ser Phe Leu His Gly Ala Ala Lys Phe Asp Thr Gln Gly Lys Thr
CAT GAA ATC TGC AGG TCC TTC CTC CAC GGT GCT GCC AAA TTT GAC ACA CAG GGC AAG ACT
          338         348         358         368         378         388
```

FIG. 3Aa.

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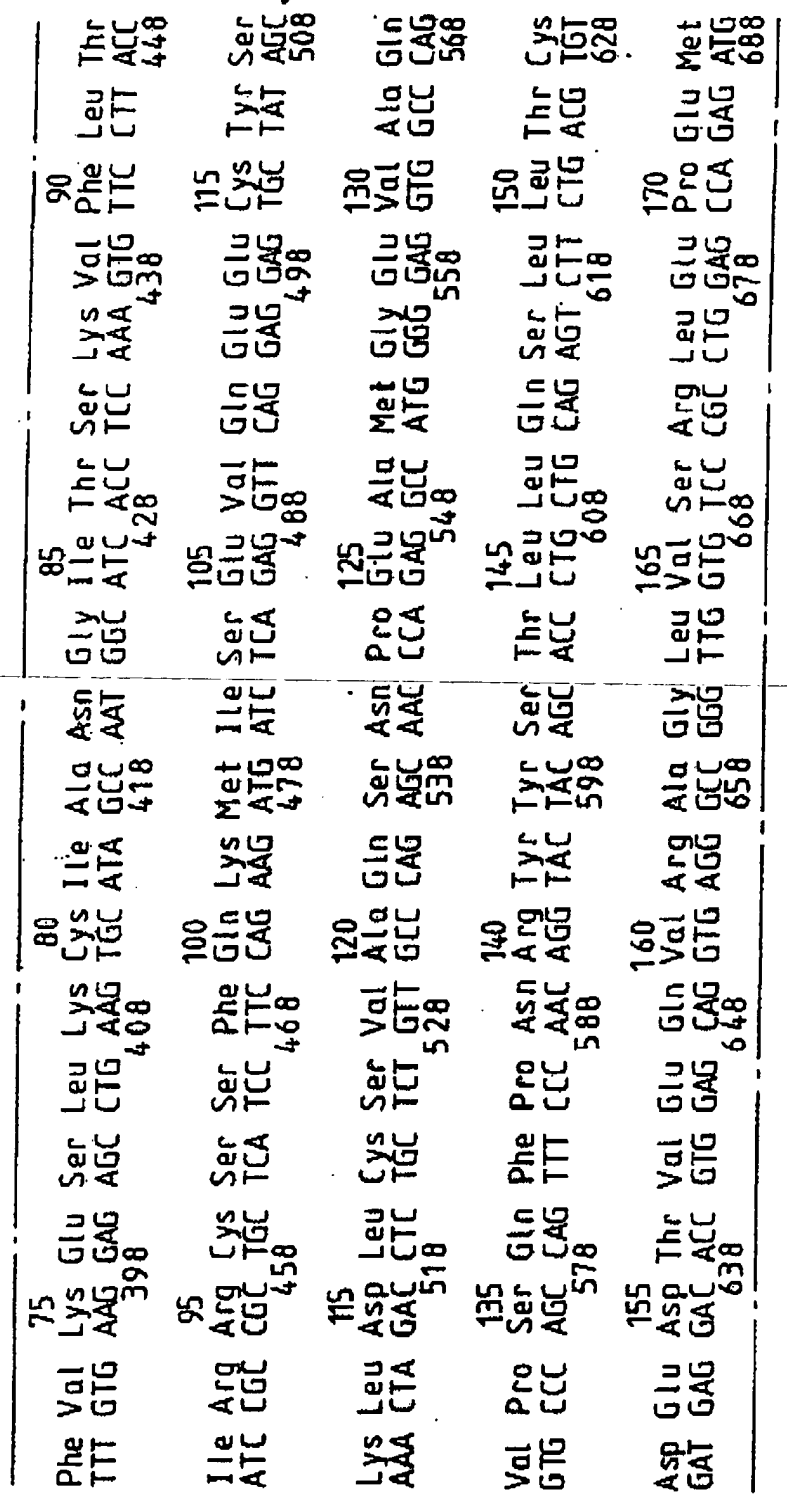
Phe Val Lys Glu Ser Leu Lys Ala Asn Gly Ile Thr Ser Lys Val Phe Leu Thr
TTT GTG AAG GAG AGC CTG AAG GCC AAT GGC ATC ACC TCC AAA GTG TTC CTT ACC
398 408 418 428 438 448
Ile Arg Cys Ser Ser Phe Ala Met Ile Ser Val Gln Glu Cys Tyr Ser
ATC CGC TGC TCA TCC TTC GCC ATG ATC TCA GTT CAG GAG TGC TAT AGC
458 468 478 488 498 508
Lys Leu Asp Leu Cys Ser Val Ala Gln Ser Asn Pro Glu Ala Met Gly Glu Val Ala Gln
AAA CTA GAC CTC TGC TCT GTT GCC CAG AGC AAC CCA GAG GCC ATG GGG GAG GTG GCC CAG
518 528 538 548 558 568
Val Pro Ser Gln Phe Pro Asn Arg Tyr Tyr Ser Thr Leu Leu Gln Ser Leu Thr Cys
GTG CCC AGC CAG TTT CCC AAC AGG TAC TAC AGC ACC CTG CTG CAG AGT CTT ACG TGT
578 588 598 608 618 628
Asp Glu Asp Thr Val Glu Gln Val Arg Ala Gly Leu Val Ser Arg Leu Glu Pro Glu Met
GAT GAG GAC ACC GTG GAG CAG GTG AGG GCC GGG TTG GTG TCC CGC CTG GAG CCA GAG ATG
638 648 658 668 678 688

FIG. 3Ab.

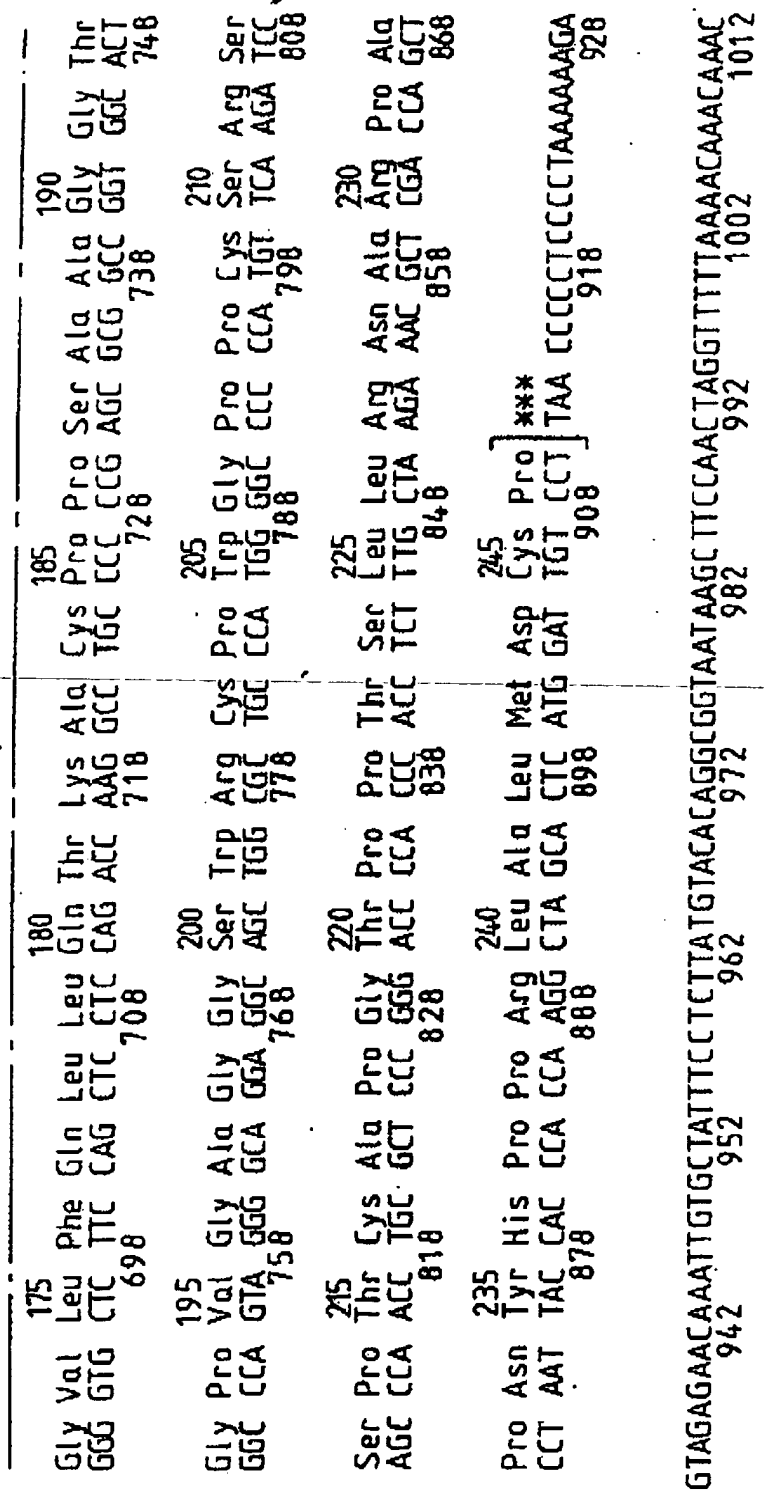
Gly Val Leu Phe Gln Leu Leu Gln Thr Lys Ala Cys Pro Pro Ser Ala Ala Gly Gly Thr
GGG GTG CTC TTC CAG CTC CTC CAG ACC AAG GCC TGC CCC CCG AGC GCG GCC GGT GGC ACT
698 708 718 728 738 748
Gly Pro Val Gly Ala Gly Gly Ser Trp Arg Cys Pro Trp Gly Pro Pro Cys Ser Arg Ser
GGC CCA GTA GGG GCA GGA GGC AGC TGG CGC TGC CCA TGG GGC CCC CCA TGT TCA AGA TCC
758 768 778 788 798 808
Ser Pro Thr Cys Ala Pro Gly Thr Pro Pro Thr Ser Leu Leu Arg Asn Ala Arg Pro Ala
AGC CCA ACC TGC GCT CCC GGG ACC CCA CCC ACC TCT TTG CTA AGA AAC GCT CGA CCA GCT
818 828 838 848 858 868
Pro Asn Tyr His Pro Pro Arg Leu Ala Leu Met Asp Cys Pro ***
CCT AAT TAC CAC CCA CCA AGG CTA GCA CTC ATG GAT TGT CCT TAA
878 888 898 908
GTAGAGAACAAATTGTGCTATTTCCTCTTATGTACACAGGCGGTAATAAGCTTCCAACTAGGTTTTTAAAACAAACAAAC
942 952 962 972 982 992 1002 1012
918 928
CCCCCTCCCCTAAAAAAGA

FIG. 3Ac.

CONTINUATION OF 3' UNTRANSLATED REGION OF THE eel CS NUCLEOTIDE SEQUENCE.

```
                                                                    1042
CTTCAGTAAACACATGTGGTCTACTAGACGACAGTATTTGATGGTGGACCTGTATTGAATGTACTAGTGTACTGGACACT
1022        1032        1042        1052        1062        1072        1082        1092

                                                                    1142
CAGCTTGTAATGTGAAGCTTTGTGTTTCTTCATGTGGTACAGTATATTCATTCTTTTTTATGTATTGCCTTGTATATCAT
1102        1112        1122        1132        1142        1152        1162        1172

                                                                    1242
CAAGTTTTGTAAGAGAATATCAGGCTGCATGGGTTTATTTTGTTGAAGGTACATTATATATCTATAAGTAATATTTT
1182        1192        1202        1212        1222        1232        1242        1252

                                                                    1342
GAGTTTAAAAATATTCGTCCTTGTACAAATCAAGGATTTTACCGAGCATAGCCAATCACACATCTTTTCATCTTTTCCCAA
1262        1272        1282        1292        1302        1312        1322        1332

                                                                    1442
ACCTCAAAGCAGATATTGAACAACATGCTCTTTGGAAATGAGGATAATCAGGATCCTGTGTTCTGATATGCAACCATAAA
1342        1352        1362        1372        1382        1392        1402        1492

                                                                    1542
GAGACCTAACAGGACGATTCTGTAGTCTTGTTAATATTTTATTGATATTTAACTTCCTAAATAATATATTTTTTCATT
1422        1432        1442        1452        1462        1472        1482        1492

                                                                    1642
TAATGTTTTTATTTGGTTGAGTTGCCATGGTTACTATTGTAAAGTACCAGGAGTTGAATGCGCAGTTCTGTTTCAGCCCT
1502        1512        1522        1532        1542        1552        1562        1572

                                                                    1642
CGTGGTGAATCTTGAAGCCCACTCAGAGGCAGTTAATTCATTATGAACAGGACATGTGCTTCGATCCCATGCTGGTCGAT
1582        1592        1602        1612        1622        1632        1642        1652
```

FIG. 3Ba.

GAGGGTCATTACAGCAAAACTATTGGCACAATTATCTGCCGGTATTTTTCCAGGGCCTTATATTAGACAGCTCAGCAACT
1662 1672 1682 1692 1702 1712 1722 1732

TGGCAAGGTTTGGAACCCTTGTAAGACAGAAAGGTCACAACATGTCAATAGTGTTACTTACTCGTGTGCCATAACACAGA
1742 1752 1762 1772 1782 1792 1802 1812

TTGATTTAGCTCTATACATTTGTTAAAAGTACAAACTTTCTGGTGGTTTGCTGTGCAAATACTACTAATAACAGGTCAT
1822 1832 1842 1852 1862 1872 1882 1892

AATAGCAAGAAGAGACTCTTCTACACCCAGCCTGATTGGCTAAGGAAACACTGTGCAGATTGTGATAGGATGCTTCATAC
1902 1912 1922 1932 1942 1952 1962 1972

TGTTGCCTGATATGCCTGATTTTAAGGCCTTAGCCAAACTCAGCAGTTGATTCTTTGTTTGCTTTCGAGCAAAAAGTTAAG
1982 1992 2002 2012 2022 2032 2042 2052

GCCTAAAGCACCTTGAAGTCAGATTCCAGAATTAACCTCAATGATTTGCTTCAAAGGACTATTGCATGTCTTTGGGGGCA
2062 2072 2082 2092 2102 2112 2122 2132

AAACCTTGGTTGAGATCGAGATAATGTCACACATTAAGAAAATGCCAAAAAAAAAACGG-3'
2142 2152 2162 2172 2182

FIG. 3Bb.

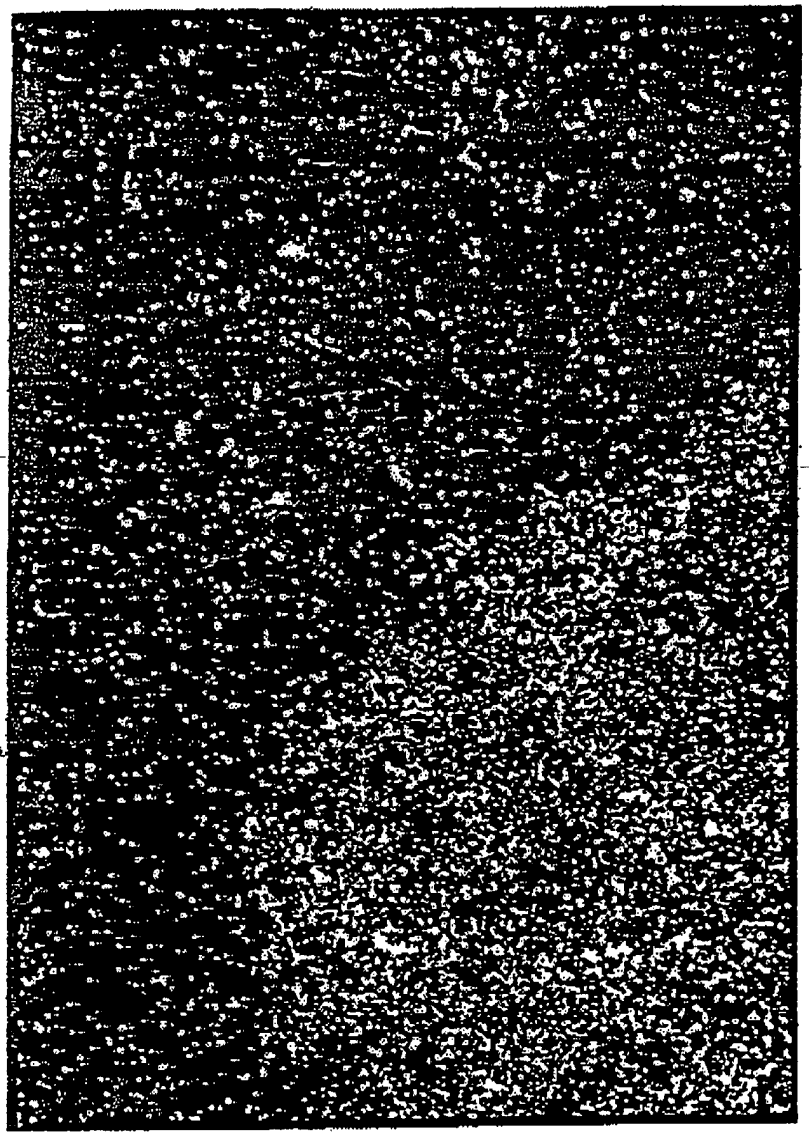

FIG. 4.

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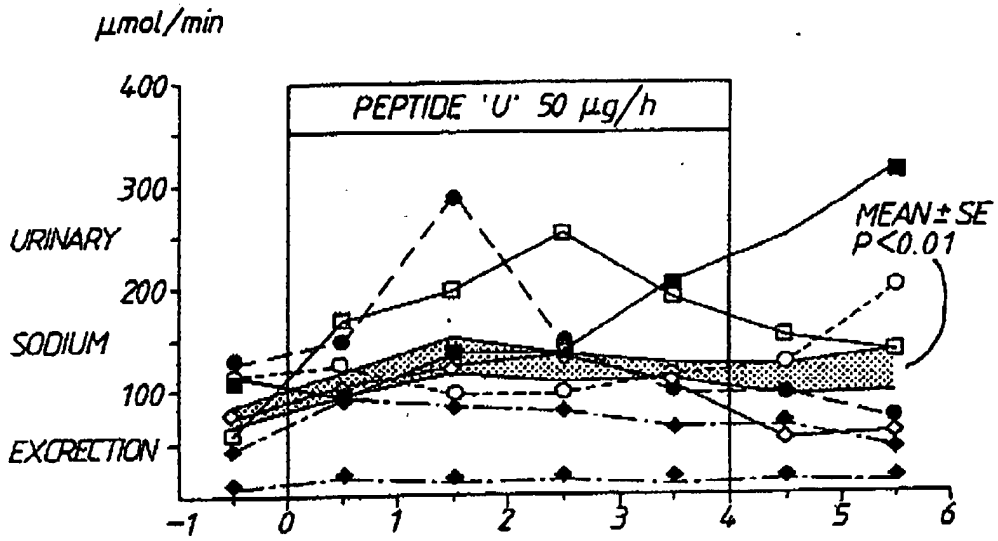

FIG. 5 (A).

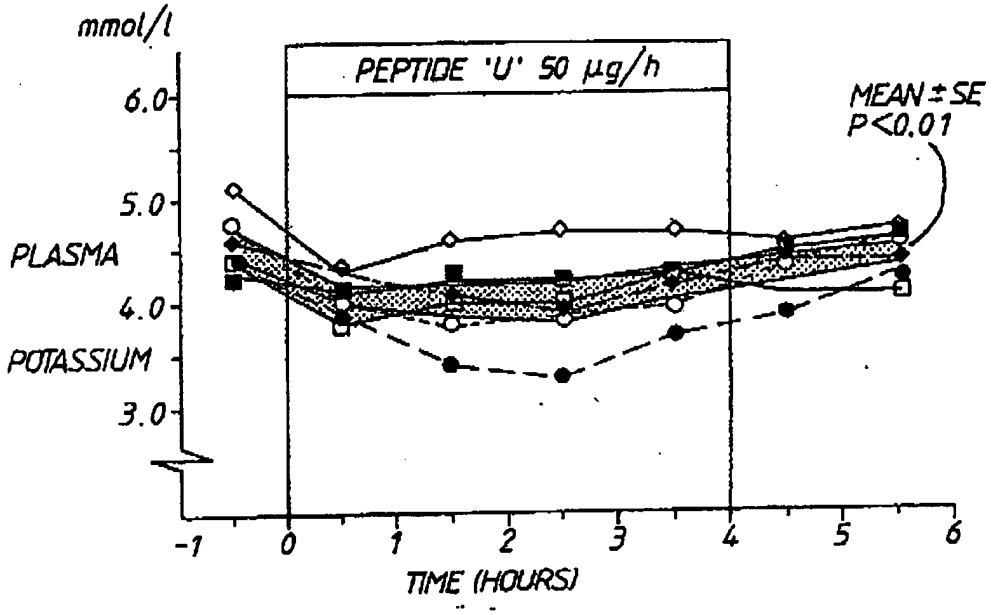

FIG. 5 (B).

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00389

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.4 C12N 15/00, A61K 37/02, C07G 17/00, C07H 21/04, C07K 3/02, 3/14, 7/08, 13/00, 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA : Keyword : Stannius (Derwent Database)

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched

AU : IPC as above, Chemical Abstracts : Keyword: Stannius, GenBank, NBRF, EMBL, Kyoto Databases

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X,Y	Wagner, G.F. et al. "Purification, Characterization, and Bioassay of Teleocalcin, a Glycoprotein from Salmon Corpuscles of Stannius", General and Comparative Endocrinology, Volume 63, issued 1986 (Academic Press, Inc., New York, USA), see pages 481 to 491.	(1-6,10-19)
X	Chemical Abstracts, Volume 99, no. 19, issued 1983, November 7, (Columbus, Ohio, USA), Ma, S.W.Y., et al. "Role of the corpuscles of Stannius and teleocalcin in calcium regulation in the teleost", see page 375, Column 1, the abstract no. 155576K, Comp. Endocrinol. Calcium Regul., Proc. Satell. Symp. 1981 (Pub. 1982) 173-179.	(1-6)
Y	Maniatis, T. et al. "Molecular Cloning : A Laboratory Manual" (1982) Cold Spring Harbour Laboratory (U.S.A.), see especially pages 226, 227, 310, 317, 329, 382, 404, 412 & 413.	(11-18)
A	Wendelelaar Bonga, S.E. et al. "Hypocalcemic Effects of Bovine Parathyroid Hormone (1-34) and Stannius Corpuscle Homogenates in Teleost Fish Adapted to Low-Calcium Water", The Journal of Experimental Zoology,	

(continued)

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7 March 1988 (07.03.88)	(14.03.88) 14 MARCH 1988
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: J.H. CHAN

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/AU 87/00389

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Volume 240, issued 1986 (Alan R. Liss, Inc., New York, USA) see pages 363 to 367

A Chemical Abstracts, Volume 103, no. 17, issued 1985, October 28, (Columbus, Ohio, USA), Takagi, Y. et al. "Effects of the removal of corpuscles of Stannius on the transport of calcium across the intestine of rainbow trout", see page 463, column 2, the abstract no. 138988e, Zool. Sci., 1985, 2 (4), 523 to 530.

(continued)

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE *

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 9 because they relate to subject matter not required to be searched by this Authority, namely:

It involves a method of treatment of the human or animal body (Rule 39).

2. [ ] Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically

3. [ ] Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING *

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (II)) (January 1985)

International Application No. PCT/AU 87/00389

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, Volume 99, no. 19, issued 1983, November 7, (Columbus, Ohio, USA), Fenwick, J.C. "Some evidence concerning the nature of the hypocalcemic factor in the Stannius corpuscles", see page 374, column 2, the abstract no. 155575j, Comp. Endocrinol. Calcium Regul., Proc. Satell, Symp. 1981 (Pub. 1982), 167 to 72.	
P,A	Harvey, S. et al. "Parathyroid Hormone-like Immunoreactivity in Fish Plasma and Tissues", General and Comparative Endocrinology, Volume 68, issued 1987, (Academic Press, Inc., New York, USA), see pages 136 to 146.	

Form PCT ISA 210 (extra sheet) (January 1985)